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(71) Applicant *(for all designated States except US)*: HYBRIDON, INC. [US/US]; One Innovation Drive, Massachusetts Biotechnology Research Park, Worcester, MA 01605 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : AGRAWAL, Sudhir [IN/US]; 61 Lamplighter Drive, Shrewsbury, MA 01545 (US). TANG, Jin, Yan [CN/US]; 16 Wells Street, #2L, Worcester, MA 01604 (US).

(74) Agent: McDONNELL, John, J.; Allegretti & Witcoff, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).

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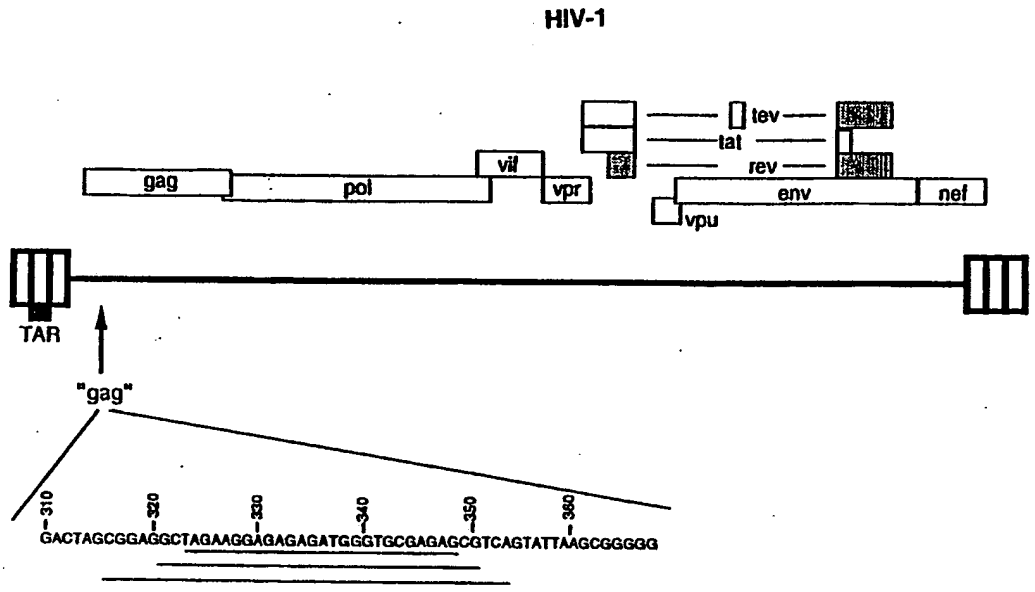

(57) Abstract

Disclosed are oligonucleotides having nucleotide sequences that hybridize to at least nucleotides 324 to 348 of a conserved *gag* region of the HIV-1 genome. These oligonucleotides have about 25 to 30 nucleotides linked by at least one non-phosphodiester internucleotide linkage which render them resistant to nuclease digestion. Also disclosed are therapeutic formulations containing such oligonucleotides and methods of inhibition HIV-1 proliferation and of treating HIV-1 infection in a mammal.

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THERAPEUTIC ANTI-HIV OLIGONUCLEOTIDE AND PHARMACEUTICAL

FIELD OF THE INVENTION

This invention relates to the treatment of HIV-1 infection. More particularly, this invention relates to chemotherapeutic agents called antisense oligonucleotides and to pharmaceutical compositions containing such oligonucleotides. This invention also relates to methods of inhibiting HIV replication and treating HIV-1 infection using such antisense oligonucleotides.

BACKGROUND OF THE INVENTION

Human T-cell leukemia lymphotropic virus-type III (HTLV-III), now more commonly known as human immunodeficiency virus type 1 (HIV-1), is thought to be the etiological agent of acquired immune deficiency syndrome (AIDS). This virus is part of the Retroviridaie family, the members of which contain an RNA genome and reverse transcriptase activity. During their growth cycle, retroviruses copy their RNA into proviral DNA. The proviral DNA is able to integrate into the chromosomal DNA of the host cell where it uses the transcriptional and translational machinery of the host to express viral RNA and proteins. Viruses are released from the cell by budding from the cytoplasmic membrane. In the case of HIV-1, viral replication results in the death of helper T-cell host cells, which leads to a state of severe immunodeficiency, to the development of various malignancies and opportunistic infections, and ultimately to the death of the infected organism.

The incidence of AIDS has risen to epidemic proportions in many countries without the development of preventative treatments or therapies which are successful in the long term. Those few therapeutic agents which have been prescribed, such as the nucleoside analogs 3'-

azido-3'-deoxythymidine (AZT), dideoxyinosine (ddI), and dideoxycytosine (ddC), have met with limited success. This has been in part because of the cytotoxicity of these agents. In addition, some viruses escape due to mutations that render them insensitive to these agents and the difficulty of antiviral action due to the ability of the virus to integrate into the host's genome. Thus, there is a long felt need for more effective therapeutic agents and preventative therapies for AIDS.

Recently new chemotherapeutic agents have been developed which are capable of modulating cellular and foreign gene expression. These agents, called antisense oligonucleotides, bind to a target single-stranded nucleic acid molecules according to the Watson-Crick or the Hoogstein rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal translation or transcription; in the case of an mRNA target, by triggering the enzymatic destruction of the message by RNase H; or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Antisense oligodeoxynucleotides have been designed to specifically inhibit the expression of HIV-1 and other viruses (see, e.g., Agrawal (1992) *Trends in Biotechnology* 10:152-158; Agrawal et al. in *Gene Regulation: Biology of Antisense RNA and DNA* (Erickson and Izant, eds.) Raven Press Ltd., New York (1992) pp. 273-283); Matsukura et al. in *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS*, Wiley-Liss, Inc. (1992) pp. 159-178; and Agrawal (1991) in *Prospects for Antisense Nucleic Acid Therapy for Cancer and AIDS*, (Wickstrom, ed.) Liss, New York, pp. 145-148). For example, it has been shown that antisense oligonucleotides having unmodified phosphodiester internucleoside bonds and sequences complementary to

portions of genomic HIV-1 ribonucleic acid (RNA) inhibit viral replication in early infected cells (Zamecnik t al. (1986) *Proc. Acid. Sci. USA* 83:4143-4147; Goodchild et al. (1988) *Proc. Natl. Acad. Sci USA* 85:5507-5511). However, these molecules are less able to inhibit viral replication in chronically infected cells (Agrawal et al. (1989) *Proc. Natl. Acad. Sci USA* 86:7790-7794), mainly because of their nuclease susceptibility (Wickstrom (1986) *J. Biochem. Biophys. Meth.* 13:97-102). Therefore, chemically modified, nuclease-resistant analogs have been developed which are effective in inhibiting HIV-1 replication in tissue cultures (Sarin et al. (1988) *Proc. Natl Acad. Sci. USA* 85:7448-7451; Agrawal et al. (1988) *Proc. Natl Acad. Sci. USA* 85:7079-7083; Matsukura et al. (1988) *Gene* 72:343-347). These analogs include oligonucleotides with nuclease-resistant phosphorothioate internucleotide linkages shown to inhibit HIV-1 replication in both acute infection (Agrawal et al. (1989) *Proc. Natl. Acad. Sci USA* 86:7790-7794) and in chronically infected cell lines (Agrawal et al. (1991) in *Gene Regulation: Biology of Antisense RNA*, eds. Erickson et al. (Raven Press, New York), pp. 273-284; Vickers et al. (1991) *Nucleic Acids Res.* 19:3359-3368; Matsukura et al. (1989) *Proc. Natl Acad. Sci.* 86:4244-4248; Agrawal et al. (1988) *Proc. Natl Acad. Sci. USA* 85:7079-7083). However, their still remains a need for a more effective anti-HIV oligonucleotide having therapeutic effects that are accompanied by low or no cellular toxicity.

SUMMARY OF THE INVENTION

Novel chemotherapeutic agents have been designed which inhibit HIV-1 replication. These agents are synthetic oligonucleotides having non-phosphodiester internucleotide linkages and a nucleotide sequence that is complementary to a portion of the conserved *gag* region of the HIV-1 genome. *Gag* is part of the structural gene of HIV-1 which is common to all retroviruses. Sequences situated around the *gag* initiation codon are known to be essential for viral packaging. The antisense oligonucleotide agent acts 6by binding to the target DNA or RNA, thereby inhibiting initiation of DNA replication and DNA expression, and inhibiting viral packaging by disrupting the secondary structure of its DNA.

Oligonucleotides of the invention are more specific, less toxic, and have greater nuclease resistance than many other chemotherapeutic agents designed to inhibit HIV-1 replication. In particular, compounds according to the invention having non-phosphodiester linkages are more resistant to nucleolytic degradation than are compounds having solely phosphodiester linkages.
In addition, they are more active in inhibiting viral replication than other known antisense oligonucleotides containing a nucleotide sequence complementary to less than the 324 to 348 *HIV-1 gag* sequence.

In its broadest aspects, the invention features an antisense oligonucleotide having a nucleotide sequence of 25 to 30 nucleotides that hybridizes to at least nucleotides 324 to 348 of the *gag* region of HIV-1. In one preferred embodiment of the invention, the oligonucleotides also have at least one phosphorothioate internucleotide linkage. Such phosphorothioate linkages contain a substitution of sulfur for oxygen, thereby

rendering the oligonucleotide resistant to nucleolytic degration.

Another embodiment of the invention is an oligonucleotide having twenty-five nucleotides linked by at least one phosphorothioate internucleotide linkage. This oligonucleotide is referred to as a "25mer." The nucleotide sequence of this 25mer is set forth in the Sequence Listing as SEQ ID NO:1. Other embodiments of the invention include phosphorothioate oligonucleotides having 26, 27, 28, 29, or 30 nucleotides, the sequences of which are complementary to nucleotides 324-348 of HIV-1 in addition to other flanking nucleotides. The sequences of two preferred 26mers are set forth in the Sequence Listing as SEQ ID NOS:2 and 3; that of a preferred 27mer are found in SEQ ID NO:4; those of preferred 28mers are found in SEQ ID NOS:5 and 6; that of a preferred 29mer is set forth in SEQ ID NO:7; and those of preferred 30mers are found in SEQ ID NOS:8 and 9.

The invention also provides therapeutic formulations including an oligonucleotide described above in a physiologically acceptable carrier, and methods of inhibiting the proliferation of HIV-1 and of treating HIV-1 infection in a mammal using these formulations.

BRIEF DESCRIPTION OF THE DRAWING

The f rgoing and other objects f this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIG. 1. is a schematic representation of the targeted *gag* initiation region of the HIV-1 genome and the complementarity thereto of antisense phosphorothioates nucleotides of the invention.

FIG. 2. is a schematic representation of the targeted *gag* initiation region and the 25mer of the invention;

FIG. 3. is a schematic representation of the targeted *gag* initiation region and the 28mer of Matsukura et al.;

FIG. 4. is a schematic representation of the targeted *gag* initiation region and the sites to be covered by antisense oligonucleotides of the invention;

FIG. 5. is a graphic representation of the HIV-1 activity described in the Tables as % inhibition of p24 expression;

FIG. 6. is a graphic representation of HIV-1 activity described in the Tables as % reduction of CPE; and

FIG. 7. is a graphic representation of a long term protection experiment, demonstrating the effectiveness of the 25mer in inhibiting p24 expression until day 17 and the ineffectiveness of ddC.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Disclosed are antisense oligodeoxynucleotides with non-phosphodiester internucleotide linkage which are particularly active in inhibiting the replication of HIV-1, and which are more resistant to nuclease digestion than oligonucleotides with phosphodiester internucleotide linkages, and which are less cytotoxic than other anti-HIV chemotherapeutic agents. These oligonucleotides (SEQ ID NOS:1-9) are targeted to the region around the *gag* initiation codon of the HIV-1 genome. Sequences situated in this region have been demonstrated to be essential for viral packaging. These sequences form a stable secondary structure (Harrison et al. (1991) in *RNA Tumor Viruses* (Coffin et al., eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 235). The oligonucleotides of the invention have been designed to bind to this region, thereby disrupting its natural stability and resulting ultimately in the inhibition of viral packaging and translation of *gag* mRNA.

The oligonucleotides are complementary to at least sequence 324-348 of the *gag* region (SEQ ID NO:11) of HIV-1 (FIG. 2) (Muessing et al. (1985) *Nature* (London) 313:450-458). Sequence 324-348 is very conserved among strains of HIV-1, as shown below in TABLE 1.

TABLE 1

	Sequence of:			
	324-348 →	TCTTCCTCTCTCTACCCACGCTCTC		
	CONSENSUS →	CGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTA		
	Strains of HIV-1	.	.	
	HTLV3/LLAV	G	A	
	HIVLAI	G	A	
	HIVNL43	G	G	
	HIVMN	G	G	
	HIVJH3	G	A	
	HIVOYI	G	A	
	HIVCDC4	G	A	
	HIVRF	G	A	
	HIVMAL	G	A	*(African)*
	HIVU455	A	A CCTCAG	*(Ugandan)*
	HIVSF2	(GA) 4G	G	
	HIVNDK	G	A	

Targeting an antisense oligonucleotide to such a conserved region including an active gene allows for efficient inhibition of HIV proliferation without the generation of "escape mutants." Escape mutants arise when a mutation occurs in a region of the genome targeted by the antisense oligonucleotide. They occur at a higher frequency in non-coding regions (like the SA region of HIV-1) than in regions encoding a protein.

The nucleotide sequences of the oligonucleotides of the invention each are complementary to at least nucleotides 324-348 of the HIV-1 genome. One aspect of the invention is an oligonucleotide consisting essentially of this sequence and is referred to herein as

a 25mer. The sequence of the 25mer is set forth in the Sequence Listing as SEQ ID NO:1. Other claimed oligonucleotid s with the ability to inhibit HIV-1 replication contain the sequence of the 25mer flanked in the 3′ and/or 5′ direction by additional nucleotides complementary to nucleotides flanking the 324 to 348 region of HIV-1. The sequence of these oligonucleotides is set forth in TABLE 2 and in the Sequence Listing as SEQ ID NOS:2-9. Also listed for comparison is the sequence of a 20mer, (Agrawal et al. (1992) *Gene Regulation: Biology of Antisense DNA and RNA* (Erickson and Izant, eds.) Raven Press, Ltd., New York, pp 273-283 (SEQ ID NO:10).

TABLE 2

Oligonucleotide	Sequence	SEQ ID No.
25mer	5′-CTCTCGCACCCATCTCTCTCCTTCT-3′	1
26mer	CTCTCGCACCCATCTCTCTCCTTCTA	2
26mer	GCTCTCGCACCCATCTCTCTCCTTCT	3
27mer	GCTCTCGCACCCATCTCTCTCCTTCTA	4
28mer	GCTCTCGCACCCATCTCTCTCCTTCTAG	5
28mer	CGCTCTCGCACCCATCTCTCTCCTTCTA	6
29mer	CGCTCTCGCACCCATCTCTCTCCTTCTAGC	7
30mer	ACGCTCTCGCACCCATCTCTCTCCTTCTAG	8
30mer	ACGCTCTCGCACCCATCTCTCTCCTTCTAGC	9
20mer	TCCTCTCTCTACCCACGCTC	10

Modified oligonucleotides of the invention are also useful inhibitors of HIV-1 proliferation, including those

with artificial internucleotde linkages other than phosph rothioate linkag s. Other known artificial linkages include methyl phosphonates, phosphoramidates, dithioates, bridged phosphorothioates, bridge phosphoramidates, sulfones, sulfates, ketos, phosphate esters, and phosphorbutylamines (see, e.g., van der Krol et al. (1988) *Biotech.* **6**:958-976; and Uhlmann et al. (1990) *Chem. Rev.* **90**:543-585). In addition, phosphorothioates and other artificial oligonucleotides having additional structures which confer nuclease resistance are also useful, such as capped 3′ and/or 5′ ends (see. e.g., U.S. Patent Application Ser. No. 07/698,568; Letsinger et al. (1989) *Biochem.* **86**:6553-6556; and Reed et al. (1991) *Bioconjugate Chem.* **2**:217-225) and chimeric oligonucleotides (see U.S. Patent No. 5,149,797, as well as hybrid oligonucleotides having regions of RNA and DNA). Other modifications conferring nuclease resistance to the oligonucleotides of the invention include 3′ terminal sequences which are internally complementary to each other, thereby forming a double stranded loop at the end of the structure. Of course other modifications of the oligonucleotides enhancing nuclease resistance, specificity, activity, and decreasing cytotoxicity, may also be performed.

The oligonucleotides of the invention can be synthesized by various known procedures including solid phase methods using phosphoramidite or H-phosphonate chemistry (see. e.g., Agrawal et al. (1992) *Ann. New York Acad. Sci.* (in press); Agrawal (1991) *TIBTECH* **10**:152-158), and can be purified by known reversed phase or ion exchange HPLC or hybridization affinity chromatographic methods (see, e.g., Metelev and Agrawal (1992) *Anal. Biochem.* **200**:342-346).

Studies of the mechanism and efficiency of antisense oligonucleotides in inhibiting viral replication can be approached effectively in several *in vitro* systems. One system uses chronically infected human T lymphocytes such as CEM cells. In such a system the infected cells are cultured in the absence and presence of different concentrations of the antisense oligonucleotide of the invention for varying lengths of time. Unlike the oligonucleotides of the invention, nucleotide analogs such as AZT, ddI, and ddC do not inhibit HIV replication in this system.

However, because chronically infected cells are CD4-, reinfection cannot occur. Thus, such an *in vitro* culture does not parallel the *in vivo* conditions present in an HIV-infected person, where only a small percentage of their CD4+ cells are infected and producing virus. A model for drug studies which more closely approaches *in vivo* condition is a cell culture with an acute, low multiplicity of infection (MOI). In this system only a fraction of the cell population harbors virus while the other cell are uninfected and are CD4+. Human T cell lines such as CEM or H9 (ATCC HTB 176) are infected with HIV for one to several hours and then cultured in the absence or presence of varying concentrations of oligonucleotide for different period of time.

The ability of the oligonucleotides to inhibit HIV-1 replication can be measured by determining the level of HIV expression and the cytotoxic effect of the oligonucleotides on the infected cells. HIV expression can be monitored by a number of parameters, including syncytia formation, p24 expression, p17 expression, and reverse transcriptase activity (see Agrawal et al (1991) *Adv. Drug Delivery Rev.* **6**:251-270; Sarin et al. (1985) *Biochem. Pharmacol.* **34**:4075-4079; and Sarin et al. (1987) *J. Natl. Cancer Inst.*

78:663-666). The inhibition of viral cryopathic effect (CPE) by the oligonucleotides can be studied by the MTT or trypan blue exclusion method.

The oligonucleotides of the invention may be used to inhibit the proliferation of HIV-1 in infected cells. In this method, a therapeutic formulation including an antisense oligonucleotide of the invention is provided in a physiologically acceptable carrier. HIV-1 infected cells are then treated with the therapeutic formulation in an amount sufficient to enable the binding of the oligonucleotide to the *gag* region of HIV-1 proviral DNA and or mRNA in the infected cells. In this way, the binding of the oligonucleotide to the HIV-1 DNA or mRNA inhibits the expression and replication of the virus.

The oligonucleotides of the invention may also be used to treat HIV-1 infection in mammals. In this method, a therapeutic formulation including an antisense oligonucleotide of the invention is provided in a physiologically acceptable carrier. The mammal is then treated with the therapeutic formulation in an amount sufficient to enable the binding of the oligonucleotide to the *gag* region of HIV-1 proviral DNA and/or mRNA in the infected cells. In this way, the binding of the oligonucleotide inhibits HIV-1 DNA expression and replication of the virus.

As used herein, a "physiologically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active

ingredients can also be incorporated into the compositions.

Effective dosages of the oligonucleotide and modes of its administration in the treatment of AIDS can be determined by routine experimentation. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms, such as bacterial and fungi. The carrier can be a solvent or dispersion medium. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents. Prolonged absorption of the injectable therapeutic agents can be brought about by the use of the compositions of agents delaying absorption.

The oligonucleotide in the carrier may be administered by intravenous or intraperitoneal injection, or by intranasal, oral, transdermal, or subcutaneous administration.

Sterile injectable solutions are prepared by incorporating the oligonucleotide in the required amount in the appropriate solvent, followed by filtered sterilization.

HIV has a high mutational rate and therefore all drugs designed to treat virus infection, including antisense oligonucleotides, may induce the formation of escape mutants. To overcome this problem, combination chemotherapy has been suggested for treatment of HIV-infected patients. This therapy involves more than one drug directed against different targets, such as reverse transcriptase inhibitors combined with protease

inhibitors. Alternatively, antisense treatment targeting different sequences either in combination or in sequential treatment schedules can be administered, resulting in different selection pressures on the virus with little time to develop escape mutants. Accordingly, the first treatments consist of a mixture of oligonucleotides of the invention, followed by sequential administration of oligonucleotides targeted to other conserved regions of the HIV-1 genome.

The invention will be further understood from the following non-limiting examples.

EXAMPLE 1

Synthesis of the Oligodeoxynucleotide Phosphorothioates.

Phosphorothioate-modified oligodeoxynucleotides are synthesized using H-phosphonate chemistry on an automated synthesizer (Millipore 8700, Bedford, MA) on a 5 to 10 mmole scale. After the assembly of the required sequence, the CPG-bound oligonucleotide H-phosphonate is oxidized with sulphur in pyridine/triethylamine/carbon disulfide to generate phosphorothiote linkages. The deprotection is completed in concentrated ammonia at 40°C for 48 hr. Purification is carried out by preparative reverse-phase chromatography followed by ion exchange chromatography. Finally, purified oligonucleotides are dialyzed against water and lyophilized. Oligonucleotide phosphorothioates are checked for their purity by HPLC and PAGE (Agrawal et al. (1989) *Proc. Natl. Acad. Sci USA* 86:7790-7794).

The oligonucleotide used for comparison in these experiments is the phosphorothioate 20mer, whose nucleotide sequence (SEQ ID NO:10) is complementary to a portion of the *gag* region (nucleotides numbers 327 - 346)

of the HIV-1 genome (see SEQ ID NO:11). Like the olig nucleotides of the invention, this 20mer has phosphorothioate internucleotide linkages. However, as shown in the experiments described in the exemplification which follows, it has less HIV-1 inhibitory activity than the oligonucleotides of the invention.

EXAMPLE 2

Specificity of Antisense and Control Oligonucleotides.

To determine the specificity of the antisense oligonucleotides, their biological effect may be compared to the same sized oligonucleotide which is not complementary to any known cellular or viral genes. Three such nonspecific control oligonucleotides are chosen, of which one having a "random" sequence is theoretically the best. The random sequence is synthesized as a degenerate oligonucleotide, by coupling a mixture of four nucleotides at each stage (theoretically it contains $4^{28} = 7.2 \times 10^{16}$ sequences), and thus measures the extent of sequence nonspecific inhibition.

EXAMPLE 3

HIV-1 Inhibition Assays.

The following assays were used to measure the ability of the oligonucleotide of the invention to inhibit HIV-1 replication.

A. Syncytia Assay

The ability of the oligonucleotides of the invention to inhibit HIV-1 replication, and thus syncytia formation, in tissue culture is tested in T cell cultures according to the method of Agrawal and Sarin (1991, ibid.) Briefly, CEM cells are infected with HIV-1 virions (0.01 - 0.1 $TCID_{50}$/cell) for one hour at 37°C. After one hour, unadsorbed virions are washed and the infected cells are divided among wells of 24 wellplates. To the infected cells, an appropriate concentration (from stock solution) of oligonucleotide is added to obtain the required concentration in 2 ml medium. The cells are then cultured for three days. At the end of three days, infected cells are examined visually for syncytium formation or stained with trypan blue or CTT for cytopathic effect determination.

B. p24 Expression Assay

HIV expression can be determined by measuring the level of viral protein p24 expression in CEM cells essentially as described by Agrawal and Sarin (*Adv. Drug Delivery Rev.* (1991) 6:251-270). Briefly, cells are pelleted and the resuspended in phosphate saline at a concentration of about 10^6 /ml. The cells are spotted on toxoplasmosis slides, air dried, and fixed in methanol/acetone (1:1) for 15 min at room temperature (RT). The slides are next incubated with 10% normal goat serum at RT for 30 min and washed with phosphate buffered saline (PBS). Anti-p24 monoclonal antibody is added to each well, and the slides are incubated in a humid

chamber at 37°C. The slides are labelled with goat anti-mouse IgG for 30 min and then washed in PBS overnight. The percentage of cells fluorescing in oligonucleotide-treated and untreated cells is compared.

C. Cytopathic Effect (CPE)

HIV-induced cytopathic effect is determined by measuring the decrease in the number of viable cells after infection. The cells are counted by adding MTT or trypan blue dye to the cells and determining how may cells (dead) take up the dye. The assay is done in triplicate.

D. Reverse Transcriptase Assay

This assay is performed essentially as described in Agrawal et al. (*Adv. Drug Delivery Rev.* (1991) 6:251-270). Supernatants from virus-infected cultures in the presence and absence of oligonucleotide are collected and virus particles precipitated with poly(ethyleneglycol). The virus pellet is suspended in 300 µl of buffer containing 50 mM Tris-HCl (pH 6.8), 5 mM dithiothreitol (DTT), 250 mM KCl, and 25% Triton X-100. Reverse transcriptase actiovity in the solubilized pellet is assayed in a 50 µl reaction mixture containing 50 mM Tris-HCl (pH 7.8), 5 mM DTT, 100 mM KCl, 0.01% Triton X-100, 5 µg dt15.rAn as template primer, 10 mM MgCl2, 15 µM [^{3}H]dTTP (15 Ci/mmol), and 10 µl of the disrupted virus suspension. After incubation for 1 hr at 37°C and subsequent addition of 50 µg yeast tRNA, the incorporation into the cold trichloroacetic acid-insoluble DNA fraction is assayed by counting in a ß scintillation counter.

EXAMPLE 3

Inhibition of HIV-1 Replication by a 25mer *in vitro*

The ability of the antisense oligonucleotides of the invention to inhibit HIV-1 infection in a number of established cell lines can be established by performing

the short term (acute infection) and long term assays described below.

A. Short Term (acute infecti n) Assays

1. In CEM Cells:

CEM cells (5 x 10^4 cells/ml) are infected with HIV-1 (HTLV IIIB strain) for 4 hours at 37°C. Infected and uninfected cells are then cultured in the presence and absence of oligonucleotide such as 25 mer or a control oligonucleotide that has no activity (e.g., a 20mer with SEQ ID NO:10) for up to 6 days at 37°C (in triplicate). The concentrations at which the 25mer is tested are 0.32 μg/ml (0.05 μM), 1.00 μg/ml (0.2 μM), 3.2 μg/ml (0.04 μM), 10 μg/ml (1.5 μM), 32 μg/ml (4 μM), and 100 μg/ml (10.5 μM). The effective concentration to cause 50% inhibition of virus replication (EC_{50}) is determined graphically. After the experiment the level of HIV-1 expression is measured by the syncytia formation assay (TABLE 3A) and the p24 expression assay (TABLE 3B). Cytotoxicity is measured by colorimetric analysis after addition of MTT to wells as described above (TABLE 3C).

TABLE 3A

Syncytia Inhibiti n Assay

Oligo-nucleotide	Conc. µg/ml	Avg.#* Syncytia	% Inhib. Syncytia	Reduct. (%)	EC_{50} µg/ml
20mer	0.32	147	0	4	1.81
	1.00	153	0	0	
	3.2	0	100	98	
	10	0	100	100	
	32	0	100	100	
	100	0	100	100	
25mer	0.32	145	6	6	1.41
	1.00	108	30	29	
	3.2	0	100	100	
	10	0	100	100	
	32	0	100	100	
	100	0	100	100	

* Average number of syncytia formed in control (infected but untreated cells) was 153.

These results demonstrate that an oligonucleotide of the invention, the 25mer, partially inhibits (30%) syncytia formation at a lower concentration (1.00 µg/ml) than does the 20mer. In addition, the effective concentration of oligonucleotide to cause 50% inhibition of virus replication (EC_{50}) was lower for the 25mer than for 20mer, indicating that the 25mer has more activity.

TABLE 3B

HIV p24 Antigen Assay

Oligo-nucleotide	Conc. µg/ml	p24 expression % of virus control	Reduct.of p24 expression (%)
20mer	0.32	133	--
	1.00	114	--
	3.20	93	7
	10	44	56
	32	53	47
	100	62	38
25mer	0.32	115	--
	1.00	115	--
	3.20	57	46
	10.00	59	41
	32	43	57
	100	35	65

The 25mer was able to reduce p24 expression by nearly 50% at a lower concentration than was the 20mer. Furthermore, at high concentrations (100 µg/ml) the 25mer was nearly twice as effective in reducing p24 expression as the 20mer, indicating that it is highly active in inhibiting HIV-1 expression.

TABLE 3C

HIV Cytopathic Assay

Oligo. nucleotide	Conc. µg/ml	Reduction in viral cytopathic effect (%)	EC_{50}
		Experiment 1	
20mer	0.32	0	7.75
	1.00	6	
	3.2	28	
	10.0	62	
	32.0	84	
	100	87	
25mer	0.32	4	2.54
	1.00	26	
	3.2	56	
	10.0	87	
	32.0	95	
	100	87	

TABLE 3C

Oligonucleotide	Conc. µg/ml	Reduction in viral cytopathic effect (%)	EC_{50}
		Experiment 2	
20mer	0.32	0	3.91
	1.00	0	
	3.2	41	
	10.0	100	
	32.0	100	
	100	100	
25mer	0.30	0	
	1.00	0	
	3.2	70	
	10.0	100	
	32.2	100	
	100	100	

TABLE 3C

Oligo. nucleotide	Conc. µg/ml	Reduction in viral cytopathic effect (%)	EC_{25}	EC_{50}	EC_{90}
		Experiment 3			
20mer	.32	6	1.36	1.84	3.20
	1.00	0			
	3.2	95			
	10.0	100			
	32.0	100			
	100	100			
25mer	0.32	0	1.29	1.75	3.01
	1.00	4			
	3.2	100			
	10.0	100			
	32.0	100			
	100	100			

The EC_{50} of the 25mer required to reduce viral cytopathic effect was significantly lower than that of the 20mer in each of three experiments performed, indicating that it has less cytoxicity than 20mer.

2. In H9 Cells:

H9 cells are infected with HIV-1 ($HTLVIII_B$ or $HTLVIII_{MN}$ strains) with 0.01-0.1$TCID_{50}$/cell for 1 hour at 37°C. $TCID_{50}$ is determined by infection of H9 cells with limiting dilutions of virus and subsequent cultures for two weeks. The cultures are prepared in quadruplicate at 10-fold dilution of HIV-1. After infection, unabsorbed virions are removed by washing. Infected cells are cultured in the presence of oligonucleotide concentrations (0.005, 0.02, 0.13, and 0.6 μM) for 3 to 4 days at 37°C. The level of HIV-1 expression is monitored by measuring p24 in supernatant with a monoclonal antibody-based p24 antigen capture test (DuPont). The results are summarized in TABLE 4. Cytotoxicity is determined by culturing uninfected cells with the 25mer for 3 to 4 days and counting the cells with a Coulter counter. The results are also shown in TABLE 4and in FIG. 5.

TABLE 4

HIV p24 Antigen Assay

Expt. #	Oligo.	Conc. µg/ml	Conc. µM	% Cell Survival	Inhibition of p24 (%)
1	25mer	25.0	2.9	0.93	90
		5.0	0.5	1.03	89
		1.0	0.1	0.94	15
		0.2	0.02	0.97	26
	AZT	0.2	0.6	0.95	90
		0.04	0.1	0.98	73
		0.008	0.02	1.04	44
		0.0016	0.005	1.08	6
2	25mer	5.0		0.93	66
		11.0		1.01	20
		0.2		1.07	21
		0.04		1.02	--
3	25mer	10.0		1.0	88
		1.0		1.0	12
		0.1		1.0	--
		0.01		1.0	--

These results show that the 25mer is more effective at inhibiting p24 expression, and thus HIV-1 replication at lower concentrations than is AZT.

3. In chronically infected CEM cells:

Chronically infected CEM cells are cultured in the presence of the 25mer at concentration of 200, 64 and 20 µg/ml. Cells are then cultured at 37°C. At 24 and 48 hours of treatment, supernatants from treated cells are removed and assayed for the level of reverse transcriptase (RT) activity as described by Sarin et al. (*J. Natl. Cancer Inst.* (1987) 78:663-666). The level is compared

to the level of RT in control untreated infected cells. The results are summarized in TABLE 5.

TABLE 5

Anti-HIV Activity of the 25mer In Chronically Infected Cells

Oligo.	Conc. µg/ml	Time (hour)	% Inhibition of of RT
25mer	200	24	92
	64		49
	20		--
	200	48	87
	64		53
	20		--

The 25mer was able to inhibit RT activity in this *in vivo* system, even after 2 days, unlike nucleotide analogs which appear to have no affect chronically infected cells.

B. Long term Infection Assays

H9 cells are infected with HIV-1 ($HTLVIII_B$) at 0.01-0.1 $TCID_{50}$/cell for 2 hours, washed to removed unabsorbed virions, diluted to 2 x 10^5 cells/ml and cultured at 37°C. Every 3 to 4 days cells are diluted to 2 x 10^5/ml then cultured in fresh medium containing 5 µg/ml (0.7 µM) of the 25mer or ddC. At the time of splitting of cells, supernatant is removed and the level of p24 expression is measured by the antigen capture assay (Dupont). Results are shown in FIG. 7 and are summarized in TABLE 6.

TABLE 6

Inhibition of HIV-1 Replication by 25mer
In Long Term Culture

	Control	25mer	ddC
concentration (μM)→	-	0.60	0.05
p24 expression (pg/ml)			
day 3	489	21 (95*)	124 (75*)
day 7	188,800	790 (99*)	10,800 (94*)
day 10	210,400	380 (99*)	17,300 (91*)
day 14	130,400	870 (95*)	60,000 (56*)
day 17	95,600	5,800 (94*)	54,000 (44*)

* % inhibition of p24 compound compare with control

These results indicate that the 25mer can inhibit p24 expression, and hence HIV-1 replication, with more efficiency than can ddC, and is much more active than ddC in the long term (> ten days). This may be because nucleotide analogs are more susceptible to nuclease digestion than are oligonucleotides with phosphorothioate linkages.

Thus, oligonucleotides of the invention are more specific, less toxic, and have greater nuclease resistance than many other chemotherapeutic agents designed to inhibit HIV-1 replication. In addition, they are more active in inhibiting viral replication than other known antisense oligonucleotides containing less than the 324 to 348 *HIV-1 gag* sequence. For example, the 20mer set forth in the Sequence Listing as SEQ ID NO:10 is less active than the 25mer of the invention. Additionally, a 28mer described by Maktsukura et al. (in *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS* Wiley-Liss, Inc.,

(1991) pp.159-178) (FIG. 3) which is complem ntary to a portion of the *gag* region overlapping region 324-348 is also much less active. This may be because the ribosome binding site (AUG) and regions flanking it are securely masked by the oligonucleotides of the invention that are at least 25 nucleotides in length. Also, when hybridized to this region, the oligonucleotides of the invention cannot be easily replaced by the ribosome, hence thwarting HIV infection. Furthermore, the conservation of this *gag* region results in the avoidance of escape mutants. This effect can be further increased by using oligonucleotides of the invention in conjunction with other anti-HIV oligonucleotides or anti-HIV drugs.

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the particular substances and procedures described herein. Such equivalents are considered to be within the scope and spirit of this invention, and are covered by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Agrawal, Sudhir

(ii) TITLE OF INVENTION: Therapeutic Anti-HIV Antiviral Oligonucleotides and Pharmaceutical Formulations Thereof

(iii) NUMBER OF SEQUENCES: 11

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Allegretti & Witcoff, Ltd.
(B) STREET: 10 South Wacker Drive, Suite 3000
(C) CITY: Chicago
(D) STATE: Illinois
(E) COUNTRY: USA
(F) ZIP: 60606

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Kerner, Ann-Louise
(B) REGISTRATION NUMBER: 33,523
(C) REFERENCE/DOCKET NUMBER: 92,623

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-345-9100
(B) TELEFAX: 617-345-9111

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE
(A) NAME: GEM 90
(B) LOCATION: complementary to bp324-348 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTCTCGCACC CATCTCTCTC CTTCT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 323-348 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTCTCGCACC CATCTCTCTC CTTCTA 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 324-349 f HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCTCTCGCAC CCATCTCTCT CCTTCT 26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 323-349 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCTCTCGCAC CCATCTCTCT CCTTCTA 27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 322-349 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTCTCGCAC CCATCTCTCT CCTTCTAG 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 323-350 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCTCTCGCA CCCATCTCTC TCCTTCTA 28

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 322-350 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCTCTCGCA CCCATCTCTC TCCTTCTAG 29

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 321-350 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCTCTCGCA CCCATCTCTC TCCTTCTAGC 30

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(B) LOCATION: complementary to bp 322-351 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACGCTCTCGC ACCCATCTCT CTCCTTCTAG 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(ix) FEATURE:
(A) NAME: GEM 90
(B) LOCATION: complementary to bp 327-346 of HIV-1 DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTCGCACCCA TCTCTCTCCT

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to genomic RNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE: HIV-1

(viii) POSITION IN GENOME: 311-380

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG

TCAGTATTAA GCGGGGGAGA ATTAGATCGA

What is claimed is:

1. An oligonucleotide having a nucleotide sequenc that hybridizes to at least nucle tides 324 to 348 of a conserved *gag* region of the HIV-1 genome, the oligonucleotide having about 25 to 30 nucleotides linked by at least one non-phosphodiester, internucleotide linkage, the linkage rendering the oligonucleotide resistant to nuclease digestion.

2. The oligonucleotide of claim 1 wherein at least one of the non-phosphodiester internucleotide linkage is a phosphorothioate linkage.

3. The oligonucleotide of claim 2 wherein the oligonucleotide has 25 nucleotides.

4. The oligonucleotide of claim 3 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:1.

5. The oligonucleotide of claim 2 wherein the oligonucleotide has 26 nucleotides.

6. The oligonucleotide of claim 5 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:2.

7. The oligonucleotide of claim 5 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:3.

8. The oligonucleotide of claim 2 wherein the oligonucleotide has 27 nucleotides.

9. The oligonucleotide of claim 9 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:4.

10. The oligonucleotide of claim 2 wherein the oligonucleotide has 28 nucleotides.

11. The oligonucleotide of claim 10 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:5.

12. The oligonucleotide of claim 10 having the nucle tide sequence set forth in th Sequence Listing as SEQ ID NO:6.

13. The oligonucleotide of claim 2 wherein the oligonucleotide has 29 nucleotides.

14. The oligonucleotide of claim 13 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:7.

15. The oligonucleotide of claim 2 wherein the oligonucleotide has 30 nucleotides.

16. The oligonucleotide of claim 15 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:8.

17. The oligonucleotide of claim 15 having the nucleotide sequence set forth in the Sequence Listing as SEQ ID NO:9.

18. A therapeutic formulation comprising the oligonucleotide of claim 1 in a physiologically acceptable carrier.

19. A therapeutic formulation comprising the oligonucleotide of claim 4 in a physiologically acceptable carrier.

20. A therapeutic formulation comprising a first and second anti-HIV-1 antisense oligonucleotide, the first oligonucleotide being the oligonucleotide of claim 1.

21. A method of inhibiting the proliferation of HIV-1 comprising the steps of:

(a) providing a therapeutic formulation comprising the oligonucleotide of claim 1 in a physiologically acceptable carrier; and

(b) treating HIV-1 infected cells with the therapeutic formulation in an amount sufficient to enable the binding of the oligonucleotide to the *gag*

region of any HIV-1 proviral DNA or mRNA in the infected cells,

whereby the binding of the oligonucleotide to the HIV-1 DNA or mRNA inhibits the proliferation of HIV-1.

22. The method of claim 21 wherein the providing step comprises providing an oligonucleotide having 25 nucleotides linked by at least one phosphorothioate linkage, the sequence of the oligonucleotide being set forth in the Sequence Listing as SEQ ID NO:1.

23. A method of treating HIV-1 infection in a mammal comprising the steps of:

(a) providing a therapeutic formulation comprising the oligonucleotide of claim 1 in a physiologically acceptable carrier; and

(b) treating HIV-1 infected cells with the therapeutic formulation in an amount sufficient to enable the binding of the oligonucleotide to the *gag* region of any HIV-1 proviral DNA or mRNA in the infected cells,

whereby the binding of the oligonucleotide to the HIV-1 DNA or mRNA inhibits the expression and replication of the HIV-1 DNA in the mammal.

24. The method of claim 23 wherein the providing step comprises providing an oligonucleotide having 25 nucleotides linked by at least one phosphorothioate linkage, the sequence of the oligonucleotide being set forth in the Sequence Listing as SEQ ID NO:1.

FIGURE 1

HIV-1 311-361
GACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAA
25mer
26mer
26mer
27mer
28mer
28mer
29mer
30mer
30mer

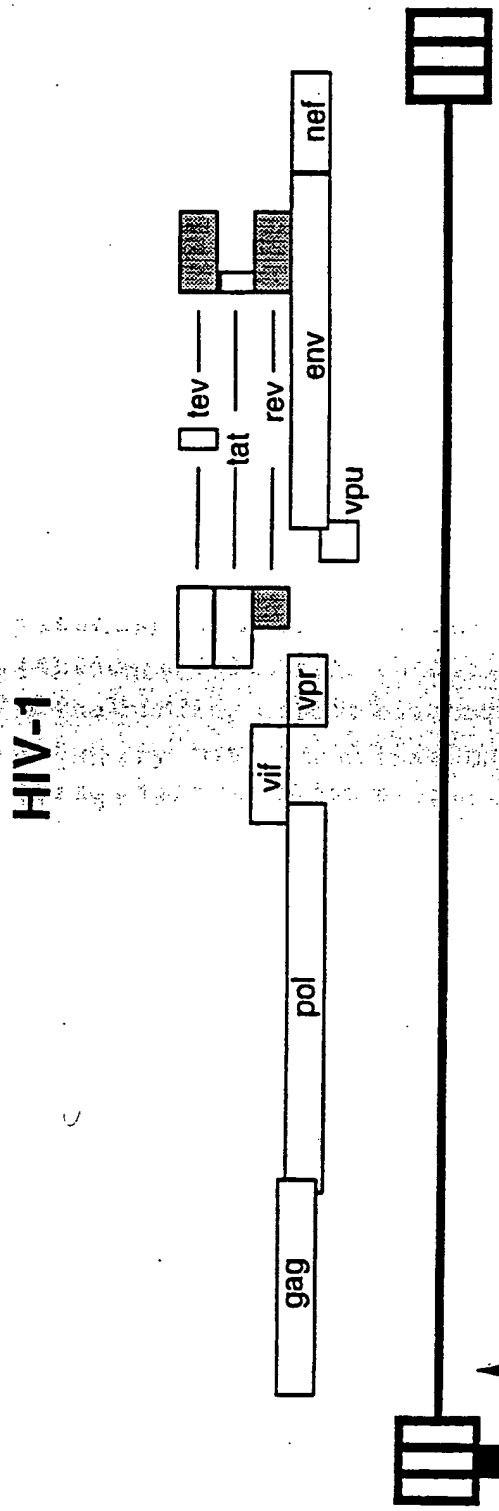
HIV-1
tev
tat
rev
env
nef
vpu
vif
vpr
pol
gag
TAR
"gag"
310
320
330
340
350
360
GACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGG
3'TCTTCCTCTCTCTACGCACGCTCTC5'
(GEM 91)

FIG. 2

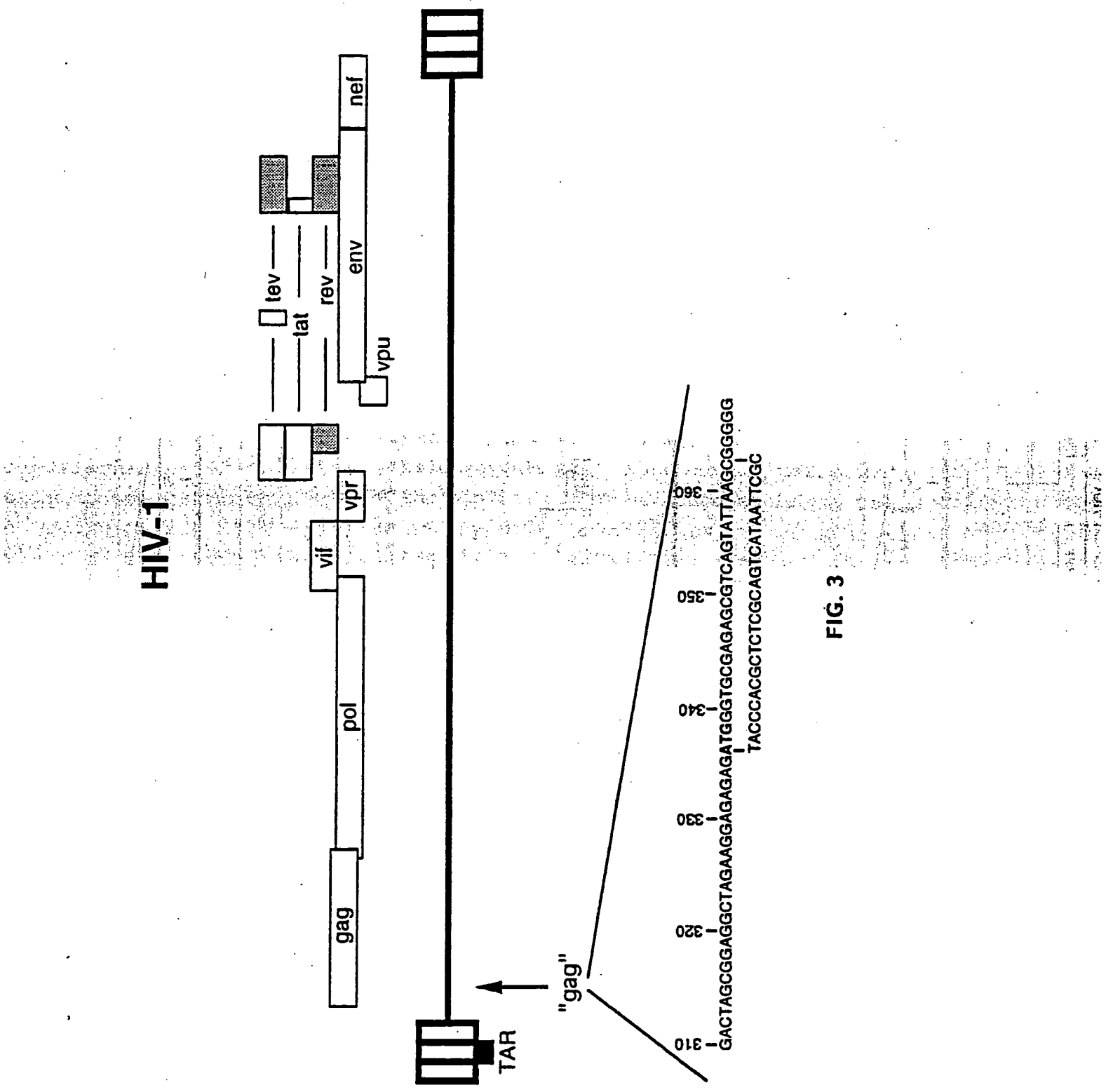
HIV-1
tev
tat
rev
env
nef
vpu
vpr
vif
pol
gag
TAR
"gag"
310
320
330
340
350
360
GACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGG
TACCCACGCTCTCGCAGTCATAATTCGC

FIG. 3

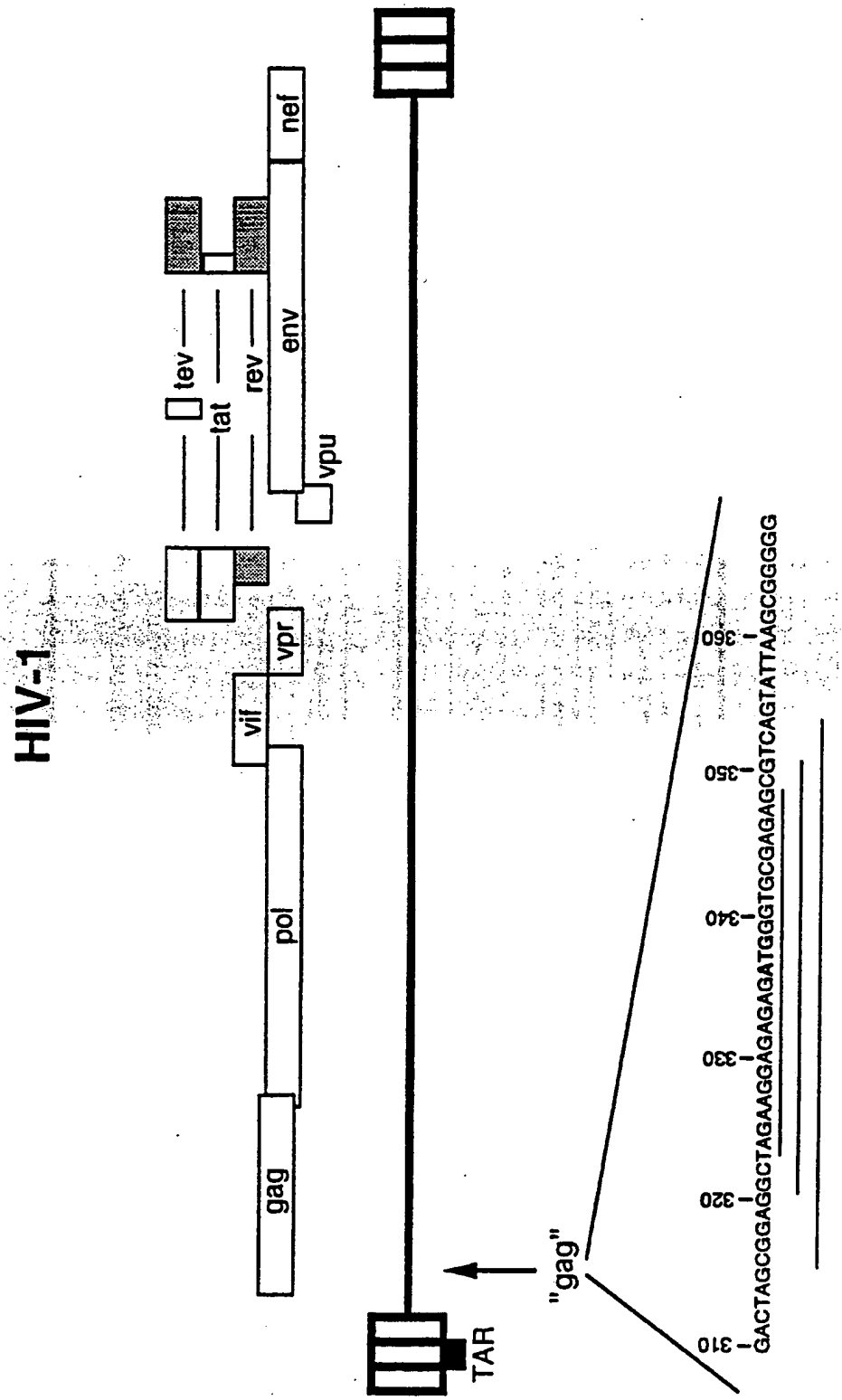
HIV-1
tev
tat
rev
env
nef
vpu
vif
vpr
pol
gag
TAR
"gag"
310
320
330
340
350
360
GACTAGCGGAGGCTAGAAGGAGAGAGATGGGTGCGAGAGCGTCAGTATTAAGCGGGGG

FIG. 4

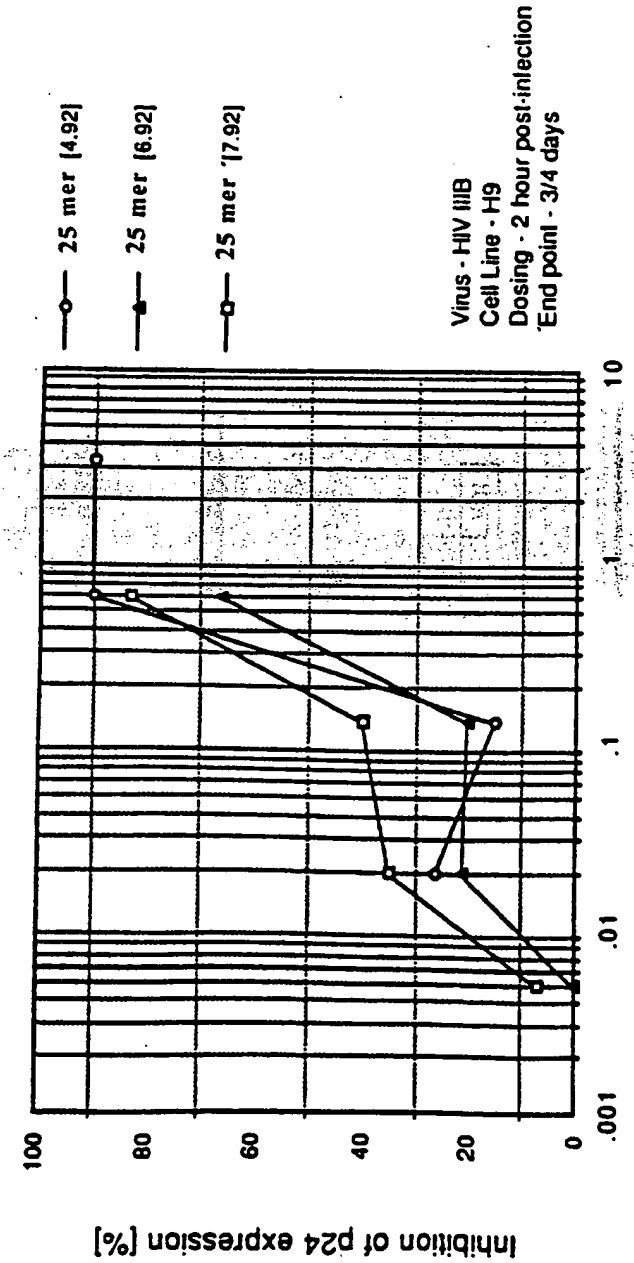
25 mer [4.92]
25 mer [6.92]
25 mer [7.92]
Virus - HIV IIIB
Cell Line - H9
Dosing - 2 hour post-inlection
End point - 3/4 days
Concentration [micromole]
10
1
.1
.01
.001
Inhibition of p24 expression [%]
100
80
60
40
20
0

FIG. 5

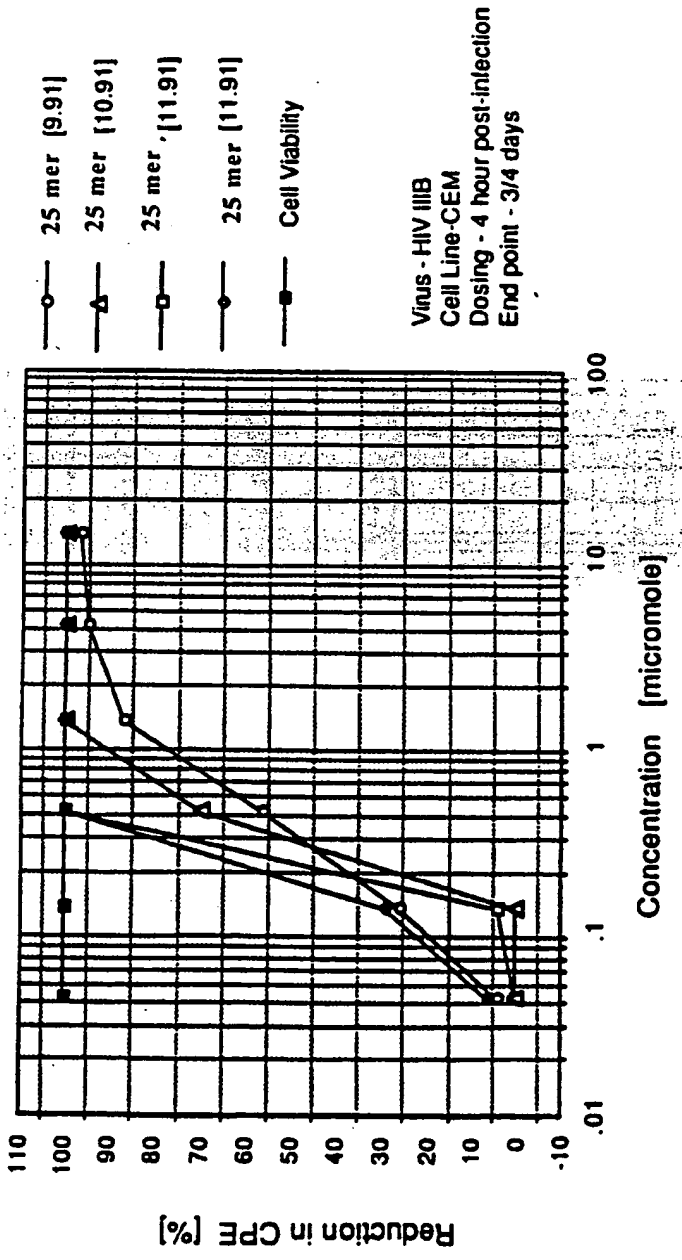
25 mer [9.91]
25 mer [10.91]
25 mer [11.91]
25 mer [11.91]
Cell Viability
Virus - HIV IIIB
Cell Line-CEM
Dosing - 4 hour post-infection
End point - 3/4 days
Reduction in CPE [%]
110
100
90
80
70
60
50
40
30
20
10
0
-10
.01
.1
1
10
100
Concentration [micromole]

FIG. 6

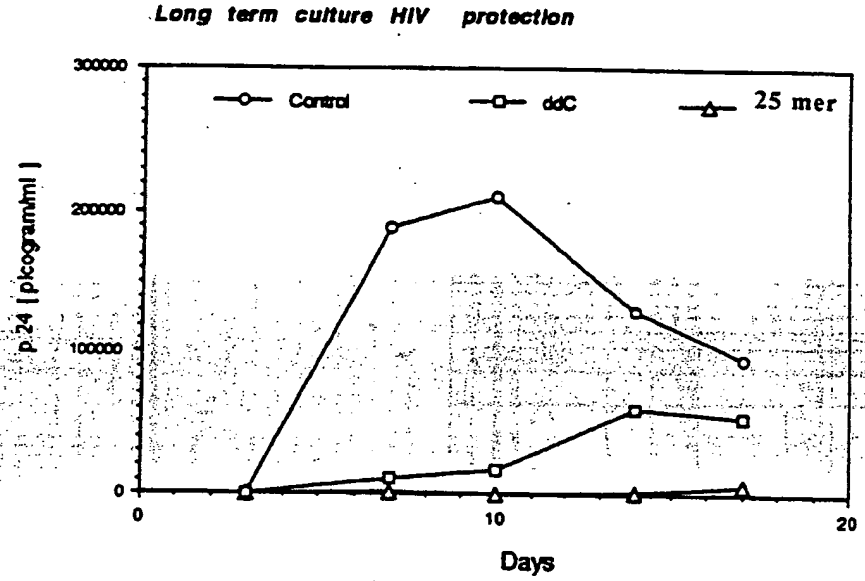
Long term culture HIV protection
300000
200000
100000
0
p24 [picogram/ml]
Control
ddC
25 mer
0
10
20
Days

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/09392

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/11 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,89 08146 (WORCESTER FOUNDATION FOR EXPERIMENTAL BIOLOGY) 8 September 1989 see page 8, line 8 - line 25 see page 15, line 11 - page 18, line 15 see page 41; table 2 see claims 2f, 3 and 4 ---	1-24
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 85 , August 1988 , WASHINGTON US pages 5507 - 5511 GOODCHILD, J. ET AL. 'Inhibition of human immunodeficiency virus replication by antisense oligodeoxynucleotides' cited in the application see table 1 --- -/--	1-24

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 1994	21 -03- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 331 939 (GREATBACH GEN-AID, LTD.) 13 September 1989 see examples 1,7 see figures 14,22	1-24
A	JOURNAL OF THEORETICAL BIOLOGY vol. 130 , 1988 pages 469 - 480 SANFORD, J. 'Applying the PDR principle to AIDS' see page 475 - page 476 see page 477, table 1, sequence 8	1
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 89 , December 1992 , WASHINGTON US pages 11209 - 11213 LISZIEWICZ, J. ET AL. 'Specific inhibition of human immunodeficiency virus type 1 replication by antisense oligonucleotides: an in vitro model for treatment' see the whole article, and especially in figure 1 the "gag" oligonucleotide	1,2,12, 18,21,23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09392

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 21-22 (as far as in vivo method are concerned) and 23-24 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort jusufying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

ernational Application No
PCT/US 93/09392

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